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NOTES

Expression and Processing of a Recombinant Human Macrophage Colony-Stimulating Factor in Mouse Cells

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A human macrophage colony-stimulating factor encoded by a 4-kilobase cDNA was expressed with bovine papillomavirus vectors in mouse cells. Pulse-chase analyses revealed that the 62-kilodalton (kDa) translation product was glycosylated, cleaved, and efficiently secreted within 1 h of synthesis. The secreted product contained both N-linked and O-linked oligosaccharide chains. Macrophage colony-stimulating factor was present extracellularly as an 80-kDa homodimer and as a multimeric species of greater than 200 kDa that may be associated with other glycoproteins.

Macrophage colony-stimulating factor (M-CSF; also termed CSF-1) is necessary for the growth and differentiation of hematopoietic cells in the mononuclear phagocytic lineage (2, 13). This glycoprotein homodimer affects growth by interactions with its cell surface receptor, encoded by the c-*fms* proto-oncogene (24). M-CSF stimulates mature macrophage and monocyte effector functions such as the production of other cytokines (14, 28), resistance to virus infection (11), and tumor cell lysis (18; A. Sampson-Johannes and J. Carlino, J. Immunol., in press). Studies also suggest a role for M-CSF during pregnancy (17, 22).

cDNAs encoding two different biologically active human M-CSFs have been molecularly cloned (8, 10, 29). A single gene on chromosome 5 (16) encodes a primary M-CSF transcript that is alternatively spliced to generate multiple mRNA species (7, 8, 10, 29). The predicted 256-amino-acid translation product of the 1.6-kilobase (kb) mRNA contains a presumptive transmembrane region at residues 166 to 188 and two potential N-glycosylation sites. The ca. 4.5-kb mRNA predicts a 554-amino-acid translation product composed of the 1.6-kb mRNA-encoded polypeptide with an additional 298 amino acids between residues 182 and 183. This larger protein contains one additional potential N-glycosylation site. The 256-residue short form and the 554-residue long form of M-CSF are processed by single peptide cleavage, glycosylation, and proteolytic cleavage to yield products of about 153 and 223 amino acids, respectively (1, 29).

Here, the biosynthesis of the long form of human M-CSF is described. In this study, bovine papillomavirus (BPV) vectors were used to express the glycoprotein in mouse C127 cells. The M-CSF translation product was rapidly processed, and an 80-kilodalton (kDa) homodimer and multimeric species of over 200 kDa were efficiently secreted.

M-CSF expression in C127 cells with BPV vectors. Three different BPV vectors were constructed to express the protein encoded by the 4-kb human M-CSF cDNA (Fig. 1). Mouse C127 cells (a mouse mammary tumor cell line, ATCC CRL1616) were transfected with expression plasmid pMY6, pMY8, or pMY11 by the calcium phosphate coprecipitation technique (3, 4). Cells harboring BPV plasmids were selected for, expanded, and propagated in medium containing 20 μM $CdCl_2$. The amount of M-CSF secreted by transformants was quantitated by a radioimmunoassay specific for human M-CSF (10). Parental untransfected C127 cells do secrete low levels of murine M-CSF (6) which does not cross-react with the anti-human M-CSF reagents used in this study. Clonal lines and populations (20 to 30 colonies) harboring pMY6 secreted 5 to 15 μg of M-CSF per ml of medium after 7 days in culture. Cells containing pMY11, which is identical to pMY6 except that it contains the eucaryotic translation consensus sequence (9), secreted M-CSF at levels similar to those of cells harboring pMY6. This suggested that the translation consensus sequence did not confer higher levels of recombinant protein expression. This is consistent with observations made by Sarver et al. (23). Cells harboring pMY8 secreted one-fifth the amount of M-CSF secreted by pMY6- or pMY11-containing cells. This suggested that the simple intronless expression units of pMY6 and pMY11 allowed more efficient expression than the complicated intron-containing expression unit in pMY8. A similar intronless expression unit was preferable to an intron-containing one for the expression of plasminogen activator (19).

One line (M66-20) containing pMY6 and one population of cells (M11-M) harboring pMY11, both secreting about 2 μg of M-CSF per ml of medium per day, were used in biosynthesis studies. The cell cultures synthesized a homogeneous M-CSF mRNA of the expected size (data not shown). M66-20 and M11-M cells gave similar results in M-CSF-processing studies; those from M66-20 cells are presented.

Processing and secretion of M-CSF. Confluent monolayers of M66-20 cells were pulse-labeled for 15 min with [^{35}S]methionine and then incubated in chase medium containing an excess of unlabeled methionine. Cells and medium were collected from parallel cultures after various times of chase. M-CSF proteins were immunoprecipitated from solubilized cell extracts or medium with a polyclonal antibody to human M-CSF produced in simian cells (5). Immunoprecipitates were subjected to digestion with various glycosidases to assess the nature of the oligosaccharide chains. Endoglycosidase H (endo H) was used to remove high-mannose immature N-linked oligosaccharides. Endo-α-*N*-acetylgalactosaminidase (endo α) was used to cleave O-

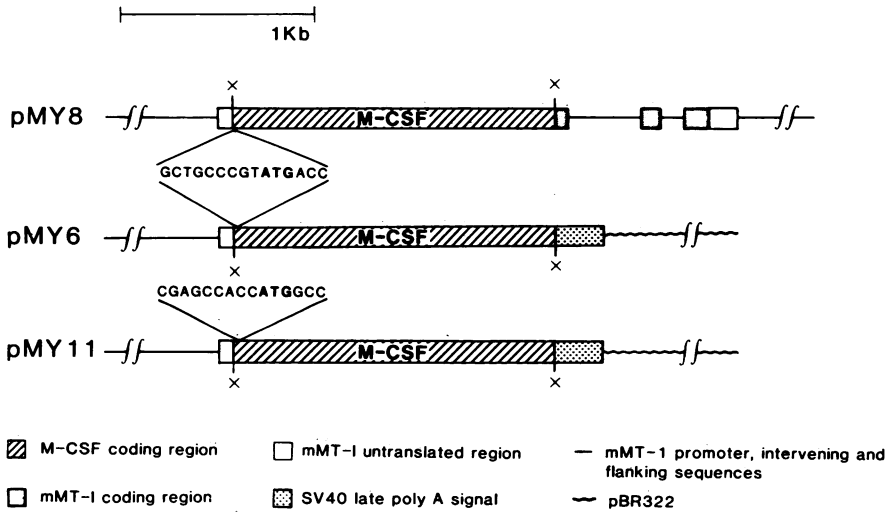

FIG. 1. M-CSF expression units in BPV vectors. pMY8, pMY6, and pMY11 were derived from BPV vector pBMT3X (15) and differ only in the cDNA expression units diagrammed here. X, *Xho*I sites. pBMT3X contains a mouse metallothionein I gene (mMT-I) which has been engineered for convenient insertion of genes of interest under control of the inducible mouse metallothionein I promoter; the human metallothionein Ia gene for selection of cells that harbor the vector; the complete BPV genome; and pBR322 sequences. pMY8 was derived by inserting a 1.8-kb *Xho*I fragment containing the M-CSF coding region and stop codon from pcDBCSF-4 (10) into pBMT3X. pMY6 and pMY11 were derived similarly from pMY5. pMY5 was derived from pBMT3X by replacing the mouse metallothionein I gene 3′ of the unique *Xho*I site with the 254-base-pair simian virus 40 (SV40) late polyadenylation signal (nucleotides 2770 to 2533 [26]). pMY11 is identical to pMY6 except that the sequence surrounding the M-CSF initiation codon have been altered to match the mammalian cell consensus sequence (9).

linked carbohydrates (27). Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions.

The analysis of N-glycosylation, processing, and secretion of M-CSF is shown in Fig. 2. Extracts from parental C127 cells did not reveal proteins that were immunoprecipitated by the M-CSF antibody (lane C, Cells). Cells treated with tunicamycin, which prevents the addition of N-linked oligosaccharide chains (25), synthesized an M-CSF precursor which migrated as a doublet of ca. 62 kDa (Fig. 2, lane 0, T). In untreated cells this initial product appeared as a 70-kDa doublet that was sensitive to endo H (Fig. 2, lanes 0, + and −), suggesting that it had been core glycosylated. At 0 (lane 0, −) and 15 (not shown) min after labeling, two ca. 50-kDa endo H-sensitive species were also apparent. Treatment with endo H reduced these intermediates to ca. 45-kDa deglycosylated species (Fig. 2, lane 0, +). Corresponding unglycosylated ca. 45-kDa species were apparent in tunicamycin-treated cells (Fig. 2, lane 0, T). Within 30 min of synthesis, 40- and 200-kDa endo H-resistant intracellular products were seen (Fig. 2, lanes 30, + and −, Cells). These products were extracellular within 15 min of synthesis, and secretion was essentially complete within 1 h (Fig. 2, Medium; see Fig. 4). Secreted M-CSF was resistant to endo H (Fig. 2, Medium, lane 60, +), indicating that it had been terminally N-glycosylated.

Analysis of the extracellular M-CSF under reducing conditions (Fig. 2, Medium) revealed a 40-kDa monomeric species, a minor 88-kDa species, and a 200-kDa multimeric species. Tunicamycin-treated cells secreted corresponding 34-, 80-, and 200-kDa species with kinetics similar to those of untreated cells. Parental C127 cells did not secrete proteins that were immunoprecipitated by the M-CSF antibody (Fig. 2, lane C). Western blot (immunoblot) analysis of the secreted products with an antibody to human M-CSF produced in bacteria indicated that both the 88- and 200-kDa species contain the M-CSF glycoprotein (12). It is likely that the

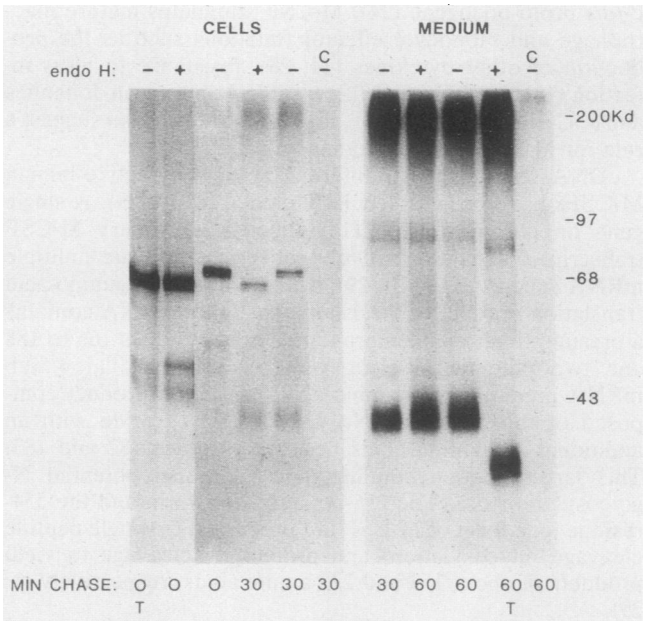

FIG. 2. Kinetics of N-glycosylation and secretion of M-CSF. M66-20 cells were pulse-labeled with [^{35}S]methionine (200 μCi/ml) for 15 min and then incubated in chase medium containing an excess of unlabeled methionine. Solubilized cell extracts or medium were immunoprecipitated with antibody to M-CSF (5). Endo H digestion was done as recommended by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and visualized by fluorography. Time of incubation in chase medium is noted at the bottom of each lane. T denotes samples from cells that were treated with 10 μg/ml of tunicamycin per ml during methionine predepletion, label, and chase. C, Samples from control C127 cells. + and −, Samples digested with endo H or undigested, respectively. Positions of molecular size markers are shown at the right (Kd, kilodaltons).

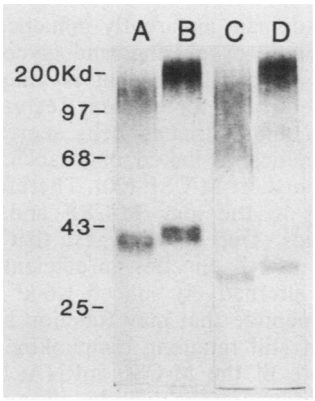

FIG. 3. Detection of O-linked oligosaccharide chains on secreted M-CSF. Medium was collected from M66-20 cells 2.5 h after pulse-labeling with [^{35}S]methionine (200 μCi/ml) for 15 min. M-CSF was immunoprecipitated as described in the legend to Fig. 2, and endo α digestion was done as recommended by the manufacturer (Boehringer Mannheim). Products were disulfide reduced and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Lanes A and B, M66-20 cells; lanes C and D, M66-20 cells treated with 10 μg of tunicamycin per ml for 1 h prior to labeling and until harvest. Lanes A and C, Endo α digested; lanes B and D, undigested. Positions of molecular size markers are shown at the left (Kd, kilodaltons).

200-kDa species contains proteins in addition to M-CSF, since the [^{35}S]methionine present in the 200-kDa secreted product far exceeded that of cell-associated M-CSF (Fig. 2; see Fig. 4). Analysis of the extracellular M-CSF under nonreducing conditions (data not shown) revealed an 80-kDa dimer and a multimeric form of over 200 kDa. The 80-kDa dimer was distinct from the 88-kDa species observed under reducing conditions. A ca. 200-kDa multimeric species is also secreted by CHO cells producing this recombinant human M-CSF (29; L. McConlogue, personal communication). Interestingly, a 200-kDa species is not secreted by simian cells expressing this recombinant M-CSF (12). Furthermore, the short human M-CSF protein does not form analogous large multimeric species when expressed in mouse cells (6, 21).

The secreted M-CSF was digested with endo α to determine whether O-linked oligosaccharide chains were present (Fig. 3, lanes A and C). M-CSFs from untreated cells (lanes A and B) and tunicamycin-treated cells (lanes C and D) were sensitive to endo α digestion, which demonstrated the presence of O-linked carbohydrate. Both the monomer and the multimeric species showed a decrease in molecular weight after digestion. The molecular weight of the monomer from untreated cells decreased by about 3 kDa after endo α digestion. This contrasts with 3T3 cell-produced human M-CSF, which requires predigestion with neuraminidase to allow digestion with endo α (20). This suggests that although 3T3 cells and C127 cells are both of murine origin, they O glycosylate this human protein quite differently. Perhaps predigestion of C127-produced M-CSF with neuraminidase would have allowed further digestion with endo α; this was not investigated.

The kinetics of O-glycosylation was also investigated by pulse-chase studies (Fig. 4). Analysis of cell extracts showed that sensitivity to endo α was unique to the 40- and 200-kDa products (Fig. 4, lanes 15, +, and 30, +, Cells) and suggested that both species contained O-linked carbohydrate chains. Neither the 70-kDa precursor nor the 50-kDa intermediates were affected by endo α (Fig. 4, lanes 0, − and +). The

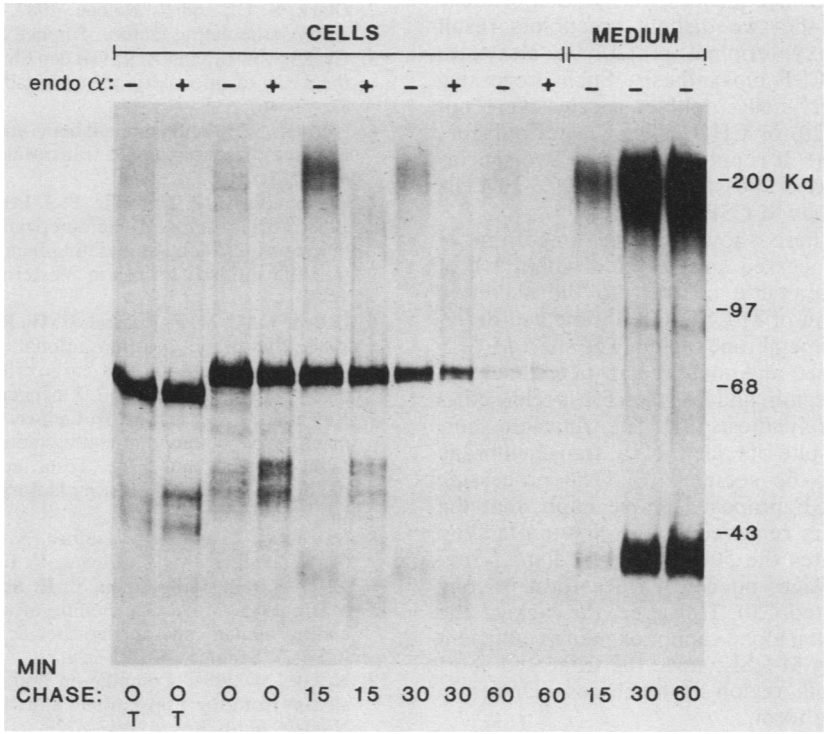

FIG. 4. Kinetics of O-glycosylation and secretion of M-CSF. Cells were labeled and immunoprecipitates were prepared from cell extracts and medium as described in the legend to Fig. 2. Endo α digestions and analyses were done as described in the legend to Fig. 3. Time in chase medium is noted at the bottom of each lane. T, Samples from cells that were pretreated with 10 μg of tunicamycin per ml for 1.5 h before labeling. +, Endo α-digested samples; −, undigested samples. Positions of molecular size markers are shown at the right (Kd, kilodaltons).

processes of final proteolytic cleavage, terminal reconstruction of N-linked sugars, and O-glycosylation appear concomitant just prior to secretion (Fig. 2 and 4).

These data suggest that the 40-kDa CSF monomer is derived from its precursor by the following processes. The 554-amino-acid initial translation product undergoes signal peptide cleavage and core N-glycosylation to produce the 70-kDa species. This product is then proteolytically cleaved to generate the endo H-sensitive 50-kDa intermediates. Terminal N-glycosylation, further proteolytic cleavage, and addition of O-linked carbohydrate generate the heterogeneous 40-kDa final product which is rapidly secreted. Alternatively, the 40-kDa monomer may be derived directly from the 70-kDa precursor, and the 50-kDa species may represent intermediates in the biosynthesis of the 200-kDa species. Analyses of the long form of human M-CSF expressed in NIH 3T3 cells (20) suggest that the 70-kDa species is rapidly dimerized and that M-CSF undergoes its subsequent processing and secretion steps as a homodimer. I assume that such dimerization occurs in C127 cells as well. Analysis of the biosynthesis of this recombinant M-CSF in CHO cells revealed intermediates very similar to those described here, including the 50-kDa species (L. McConlogue, personal communication).

Four distinct M-CSF precursor species were observed, a ca. 62-kDa doublet and a ca. 70-kDa doublet (Fig. 2 and 4). It is tempting to speculate that the lowest-molecular-weight band represents the unglycosylated peptide and that the three higher-molecular-weight species represent core glycosylation at one, two, and three of the N-glycosylation sites. However, neither tunicamycin treatment of the cells nor digestion with endo H resolved the lower doublet into one distinct band (Fig. 2 and 4). This suggests that the 62-kDa doublet represents two distinct unglycosylated species and that the 70-kDa doublet reflects core glycosylation of those two species. Possibly, the two distinct precursors result from differential carboxyl-terminal proteolytic cleavages that occur early in M-CSF biosynthesis. Such processing could be unique to C127 cells; doublet species were not observed in 3T3 cells (20) or CHO cells (L. McConlogue, personal communication). It is unlikely that the two species are derived from different messages, since M66-20 cells synthesize a homogeneous M-CSF mRNA.

The data presented here show that the long form of M-CSF is efficiently processed and secreted within 1 h of synthesis. This is in dramatic contrast to the secretion kinetics of the short form of M-CSF and a truncated derivative that lacks the transmembrane region. The short M-CSF homodimer is externalized and anchored at the cell surface by the transmembrane region, and less than 5% is released as a soluble factor 6 h after synthesis (21). The truncated short M-CSF derivative, despite its lack of a transmembrane region, requires 6 h to be secreted (6). The processing schemes for long M-CSF proposed above imply that the transmembrane region is removed in the first proteolytic cleavage, which generates the 50-kDa intermediates. Suggestive of such a cleavage, no cell surface form of long M-CSF has been detected (20). However, the lack of the membrane-spanning region alone cannot explain the efficient secretion of mature long M-CSF, since the truncated short M-CSF which lacks this region is much less efficiently secreted than is the long form.

The short and long human M-CSF proteins differ only in a 298-amino-acid region that is unique to the long protein. Interestingly, 30% of these unique amino acids are either proline or serine. With its primary sequence or the structure it induces, this domain apparently influences the secretion kinetics, proteolytic processing, and glycosylation of this M-CSF. The N-glycosylation that is unique to the long protein is not essential for efficient secretion, since tunicamycin-treated and untreated cells secreted the M-CSF with similar kinetics. O-linked oligosaccharides have not been detected on short M-CSF (20). Therefore, O-glycosylation is unique to the long M-CSF and may affect its secretion kinetics. Our data suggest that the ca. 4.5-kb human M-CSF mRNA encodes an efficiently secreted glycoprotein. The alternatively spliced 1.6-kb M-CSF mRNA encodes a polypeptide that may function as a cell surface molecule, albeit still retaining lymphokine functions (21). Perhaps as more of the M-CSF mRNAs are cloned and expressed, additional roles will be discovered for these factors.

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